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RESEARCH ARTICLE

ASSOCIATION OF ADIPONECTIN GENE POLYMORPHISM rs266729 WITH ADIPONECTIN LEVELS IN TYPE 2 DIABETIC PATIENTS WITH AND WITHOUT CVD. IN AL-NAJAF GOVERNORATE, IRAQ.

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Abstract

Background:- Cardiovascular diseases are a group of disorders of the heart and blood vessels. Adiponectin is an adipocyte-secreted protein with insulin-sensitizing and anti-atherogenic properties. Many studies demonstrated that polymorphisms within the adiponectin gene could be associated in T2DM with and without CVD.

Methods:- A case-control study was conducted to find the association between SNP rs266729 in T2DM with and without CVD in Al-Najaf Governorate, Iraq. The study included 203 T2DM patients with CVD randomly selected based on World Health Organization (WHO) guideline and 133 T2DM patients without CVD as controls group. DNA was extracted from blood and genotyped by PCR-RFLP by using (Hha1) enzyme. Multinomial logistic regression was applied to compare the proportions of genotypes and alleles. The odds ratio for risk of developing CVD in T2DM was calculated with and without adjustment for age, sex, and BMI.

Results:- The frequency of G allele of rs266729 (C/G) polymorphism was significantly lower (p=0.0001) in T2DM with CVD (27.3%) compared to that without CVD (33.08%). The homozygous genotype (GG) significantly (0R=7.570, CI 95%(1.597-32.530), P= 0.011) increased the risk of T2DM with CVD seven folds with respect to those of the wild type (CC) after adjustment age, sex, and BMI, furthermore the heterozygous (CG) genotype significantly [0R=2.818,CI 95%(1.00-12.596), P= 0.050] raised the risk of T2DM with CVD by two folds. Homozygous and heterozygous genotypes of rs266729 polymorphism exhibited significant association with decreased adiponectin levels (P= 0.08).

Conclusion:- Adiponectin gene polymorphism rs266729 is involved in the pathogenesis of T2DM with CVD. In addition this SNP may play a role in the development of cardiovascular diseases and metabolic syndrome by affecting HDL and adiponectin levels.

Background:-

Cardiovascular disease (CVD)) is the most common complication of type 2 diabetes mellitus (T2DM), and approximately (70-80%) of patients with T2DM will die of cardiovascular causes (Shah et al. 2015). Type 2 diabetes mellitus (T2DM), represents 90-95% of the total diabetes cases, ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretary defect with insulin resistance (International Diabetes Federation IDF. 2012). Important to investigate for causes and risks of T2DM, one category of these causes is genetic factors and advances such as the development of genome- wide association studies (GWAS) have been enabled the identification of a number of genes associated with T2DM risk. One of

these genes is the adiponectin gene and several studies investigated the SNP of this gene in different populations. Common genetic variants is rs266729 which is located in the promoter region of ADIPOQ, and consists of a C > G substitution in the – 11377 position rs266729 (Briefed. 2012).

Adiponectin, an adipose tissue secreted protein, has been well recognized to exhibit insulin-sensitizing, anti-inflammatory and anti-atherosclerotic properties (Prakash J. 2015). Adiponectin acts through three adiponectin receptors, ADIPOR1, ADIPOR2 and T-Cadherin. The activation of ADIPOR1 and ADIPOR2 results in increased hepatic and skeletal muscle β- oxidation of fatty acids, increased lactate production of skeletal muscle, decreased hepatic gluconeogenesis , increased glucose uptake by the cells and inhibition of oxidative stress and inflammation (Li P et al. 2015, Yamauchi et al. 2007). Activation of T-Cadherin is protective in vascular endothelial cells against oxidative stress- induced apoptosis (Joshi et al. 2005) and is strongly expressed in regions of atherosclerosis (Takeuchi et al. 2007).

Adiponectin varies according to body mass index with lower levels in obese individuals (Lang et al. 2011), its levels are low in T2DM (Pyrzak et al. 2010), consequently low levels of plasma adiponectin might play a role in the etiology of insulin resistance and T2DM (Chiodini et al. 2010, Gong et al. 2010).

Methods:-

Study design:-

A case–control study of 336 persons included two groups (203 type 2 diabetic patients T2DM with cardiovascular disease CVD and 133 type 2 diabetic patients T2DM without cardiovascular disease CVD) randomly selected was conducted to assess the association of SNP rs266729 of adiponectin (adipoQ) gene in Al-Najaf Governorate.

The study was performed on 336 type 2 diabetic patients (184 male and 152 female).The patients included two groups { 203 diabetic patients with CVD (117 male and 86 female), the patients ages ranged between (45-56) years with mean ± SD (49.03 ± 6.41) } and {133 diabetic patients without CVD (67 male and 66 female), the patients ages ranged between (41- 46) years with mean ± SD (47 ± 6.11) } were included as a control group}. The patient population who attended diabetes center at Al- Sader Medical City, Najaf, Iraq from September 2014 to January 2015. All patients were diagnose by specialist physicians as having type 2 diabetes, were based on WHO guidelines.

Phenotype Measurements:-

We collected clinical data, such as weight, height, and other data. The BMI was calculated as weight (in kg) divided by the square of height (in m). Serum cholesterol, serum triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were measured. Total serum adiponectin was estimated using an enzyme-linked immune sorbent assay (ELISA).

Genetic Analysis:-

Blood samples of T2DM with and without CVD were collected in EDTA-anticoagulant tubes, and then DNA was extracted from whole-blood samples using DNA extraction kit (Favorgen, Tawan). Then DNA concentration and purity were measured by UV absorption at 260 and 280 nm (Bio Drop, U.K.).

Genotyping was performed using polymerase chain reaction-restriction fragments length polymorphism technique (PCR-RFLP) for adiponectin gene using thermocycler (Biometra, Germany). The primer sequences were obtained from (Malgorzata et al. 2012): forward 5'GCTCTGTGTGGACTGTGGAG3' and reverse 5'AGAAGCAGCCTGGAGAACTG3'. Amplification was performed in a total volume of 25 µl which contained 12.5 µl of GOTaq Green Master Mix, (Promega Corpration, Madison, WI), 1.5 µl of each primer (1 Mm final concentration) (One Alpha, U.S.A.), 3.5 µl nuclease free water, and 6 µl of DNA template. Cycling conditions were 94 C° for 5 min followed by 40 cycles of 94 C° for 30s, 56 C° for 30s, 72 for 30s, and a final extension of 72 C° for 5 min. Amplification product of adiponectin gene was 302 bp. The product was digested with 10 U of restriction enzyme (HhaI) (Promega) and ran on 2% agarose gels.

Statistical analysis:-

Data are presented descriptively as means and standard deviation (M ± SD). Comparisons between groups at baseline were made using a two-sample t-test. Significant differences in continuous variables among more than two

groups were confirmed by the ANOVA test, using Megastat excel 2013 software. Values of P (two-tailed) less than 0.05 were considered statistically significant for all of the statistical analysis.

Genotyping and allele frequencies of rs266729C>G in Adiponectin gene, was compared in groups using a $\chi 2$ test. Odds ratio (OR) and confidence intervals (CI) were calculated to assess the relative risk conferred by a particular allele and genotype. Odds ratio, were calculated using MedCalc for windows, version 7.4.1.0 (MedCalc software, Mariakerke, Belgium). Genotype frequencies were tested for Hardy-Weinberg equilibrium using online software web-Assotest (www.ekstoem.com). Genetic power was calculated using the online software OSSE (OSSE.bii.a-star.edu.sg). Genotype and allele frequencies in with and without CVD in T2DM patients were tested by multinomial logistic regression analysis with and without adjustment for age, sex and BMI using SPSS.

Results:-

Anthropometric and biochemical characteristics of study individuals are presented in (table 1). Results of digestion with restriction enzyme (Hha1) for adiponectin gene rs266729 included 302 bp band for wild type (CC) genotype, for the heterozygous genotype (CG) three bands 302, 182 and 120 bp and for homozygous genotype (GG) two bands 182 and 120 bp as shown in (Fig. 1). Genotype and allele frequencies of adiponectin gene are shown in (table 2).

Genotype frequencies of rs266729 were consistent with Hardy-Weinberg equilibrium in both T2DM with CVD individuals (p=0.519) and T2DM witout CVD individuals (p=0.303). The power of this study to detect a significant difference at level of 0.05 was 86.9%. The results shown that adiponectin gene polymorphism rs266729 (homozygous GG and heterozygous CG genotype) was significantly associated with CVD in T2DM patients and the frequency of G allele was higher in T2DM with CVD patients.

Biochemical characteristics of T2DM with and without CVD individuals according to adiponectin gene polymorphism rs266729 genotypes are shown in(tables 3 and 4). It demonstrated that a significant impact on HDL and adiponectin levels was detected for rs266729.

Table 1

Anthropometric and biochemical characteristics of study individuals.

	T2DM with CVD subjects n=203	T2DM without CVD subjects n=113	p- value
No (M/F)	203(117/86)	133(67/66)	-----
Age (y)	49.03±6.41	47.33±6.11	0.016
BMI (Kg/m^2)	30.79±5.31	28.94±5.02	0.002
Cholesterol (mg/dl)	232.99±35.29	233.73±34.97	0.851
Triglycerides (mg/dl)	229.89±39.69	226.96±40.58	0.515
VLDL(mg/dl)	45.97±7.93	45.39±8.11	0.515
LDL (mg/dl)	139.58±35.90	139.50±37.20	0.984
HDL (mg/dl)	47.42±6.54	48.83 ±6.78	0.062
Adiponectin (ng/ml)	6.39 ±2.41	7.50 ±1.55	0.000

*Phenotypic data expressed as mean± standard deviation.

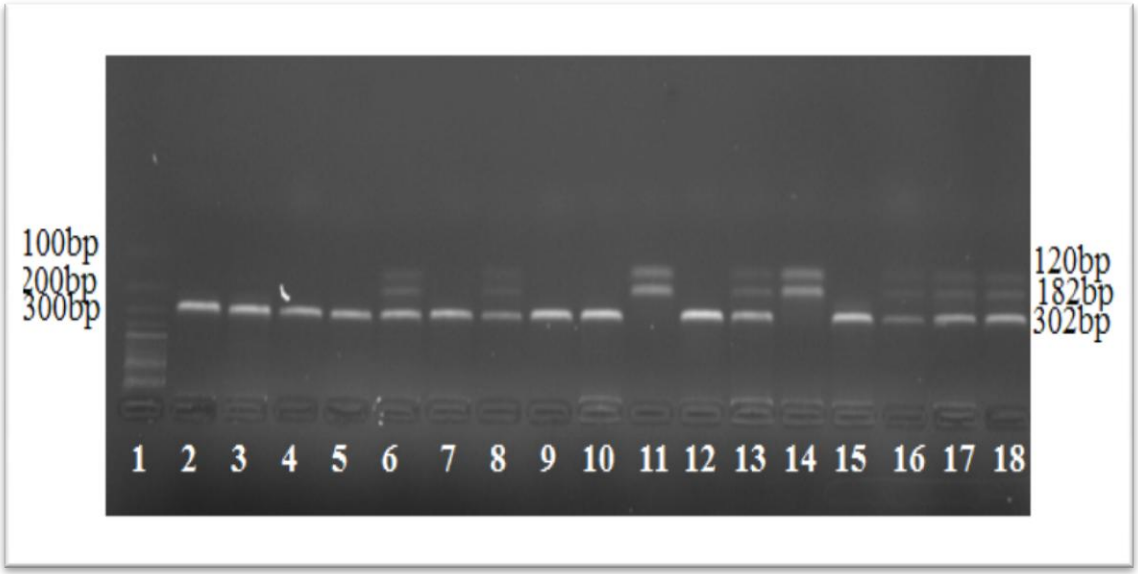

Fig. 1. Genotyping result for adiponectin gene rs266729. Marker Line 1. CC genotype 302bp. Lines 2, 3, 4, 5, 7, 9, 10, 12 and 15. Lines 6, 8, 13, 16, 17, and 18, CG genotype 302, 182, 120 bp. Lines 11 and 14: GG genotype 182, 120 bp.

Table 2

Genotype and allele frequency of rs266729 polymorphism of adiponectin gene and association of this variant in T2DM with and without CVD in the study individuals.

	T2DM with CVD n= 203	T2DM without CVD n=133	Unadjusted OR(95%CI) P-value	Adjusted OR(95%CI) P-value
Genotype				
rs266729 (C/G)				
CC	109	91	Reference	
CG	77	40	2.607(1.002-2.578) 0.049	2.818(1.00-12.596) 0.050
GG	17	2	6.045(1.555-31.016) 0.010	7.570(1.597-32.530) 0.011
Frequency of G allele	111 (27.3%)	44 (33.08%)	2.44(1.54-3.84) 0.0001	

Table 3

Biochemical characteristics of T2DM with CVD individuals according to adiponectin gene polymorphism (rs266729) genotype

Biochemical Characteristics	CC (n=109) Mean ± SD	CG(n=77) Mean ± SD	GG(n=17) Mean ± SD	P-value
BMI (Kg/m^2)	30.68±5.18	30.85±5.17	31.20±6.65	0.92
Cholesterol (mg/dl)	231.43±36.50	234.63±34.22	235.48±3.49	0.79
Triglycerides (mg/dl)	230.03 ±39.20	226.74 ±39.33	243.27±41.57	0.29
VLDL (mg/dl)	46.00±7.84	45.34±7.86	48.65±8.31	0.29
LDL (mg/dl)	137.99±37.30	141.76±34.40	139.84±6.53	0.77
HDL (mg/dl)	47.45±6.68	47.52±6.33	46.84±6.53	0.92
Adiponectin (ng/ml)	6.05±2.38	6.83±2.36	6.55±2.45	0.08

Table 4
Biochemical characteristics of T2DM without CVD individuals according to adiponectin gene polymorphism (rs266729) genotype.

Biochemical characteristics	CC (n=91) Mean ± SD	CG(n=40) Mean ± SD	GG(n=2) Mean ± SD	P-value
BMI (Kg/m^2)	29.20±4.98	28.66±3.98	22.95±2.99	0.19
Cholesterol (mg/dl)	235.08±32.91	228.95±38.81	267.78±14.57	0.24
Triglycerides (mg/dl)	228.68 ±41.95	220.55 ±36.06	276.68±0.90	0.12
VLDL (mg/dl)	45.73±8.39	44.11±7.21	55.33±0.18	0.12
LDL (mg/dl)	139.83±36.92	137.30±37.57	168.8±11.06	0.49
HDL (mg/dl)	49.51±6.86	47.54±6.42	43.65±3.33	0.16
Adiponectin (ng/ml)	7.39±1.54	7.72±1.55	8.18±0.34	0.43

Discussion:-

At the beginning, the results were analyzed for the Hardy–Weinberg equilibrium and found to be consistent with it, indicating that the investigated allele frequencies are constant between generations. These findings were also reported by Mtiraousi et al. (2012) and Alkhateeb et al. (2013).The genetic power was assessed for the adiponectin gene polymorphisms to reveal significant variation at level of (0.05) and found to be (86.9%) for -11377C>G (rs266729) SNP. These values are considered to be appropriate to achieve creditable results. However, the inability to achieve a value of 80% for the genetic power may be due to small sample size (Ellis and Paul et al. 2010; Kaftan and Hussain, 2015; HapMap.org.).

The results of adiponectin gene polymorphism rs266729 demonstrated that homozygous genotype (GG) carries have seven fold increased risk of developing CVD when compared with those of the wild type (CC) after adjustment for age, sex, and BMI in addition to the risk in heterozygous (CG) genotype carriers was two folds. Such observations strongly suggested a role of adiponectin gene polymorphism rs266729 in the pathogenesis of CVD in patients T2DM in Al-Najaf Governorate, Iraq. The current findings are consistent with results of (Kaftan and Hussain, 2015) in Iraqi population, and they are also in agreement with data of studies of European (Gable et al. 2007), and Caucasian (Prior et al. 2009).

The minor allele frequency (MAF) of rs266729 in T2DM without CVD (33.08%) was observed to be higher than the global minor allele frequency (25%) (WWW.Hapmap.Org) and lower European (38%) and Caucasian. This is possibly due to small size or due to ethnic variations (Ellis, 2010).

To realize the impact of adiponectin gene polymorphism rs266729 in the development of CVD in patients T2DM, we have to focus on the molecular mechanisms. In another study the minor allele G of rs266729 was found to alter the DNA sequence of the SP1 binding site of the transcription elements, resulting in a reduction in transcriptional activity of the adiponectin gene promoter. Thus , negatively regulating the expression of adiponectin gene leads to low plasma concentrations of adiponectin and susceptibility to T2DM and obesity (Suriyaprom et al. 2014).

Several studies reported that an increase/decrease of adiponectin levels has been observed to cause increased fat burning, decreased fatty acid content, decreased glucose level in blood circulation, and improved insulin sensitivity (Tsubakio-Yamamoto et al. 2012; Kenneth et al. 2014; Li et al. 2009). Thus , an appropriate level of adiponectin is essential to maintain normal tissue and body metabolism.

The analysis of phenotypic data stratified to the genotypes (CC, CG, and GG) of rs266729 polymorphism pointed out an interesting observation that HDL levels were found in carriers of the (GG) genotype, while the highest values were observed in those of the (CC) genotype. Inversely, the highest adiponectin magnitudes were noticed in carriers of (GG) carriers and lowest values were found in those of (CC) carriers.

Regarding the inverse relationship of HDL levels with rs266729 polymorphism and to our knowledge, it is the first investigation at which such observation was reported, Zadjali et al. 2013, and Sun et al. 2008, have studied this relationship and found no association. So how does adiponectin affect HDL levels? Adiponectin likely promotes HDL formation. First, adiponectin promotes mitochondrial fatty acid oxidation and a decrease in circulating levels of fatty acids. High serum levels of fatty acids are inhibitory to lipoprotein lipase. Reducing fatty acid concentrations would remove the biochemical brake applied to lipoprotein lipase. Secondly, adiponectin activates peroxisome

proliferator- activated receptor-α (PPAR-α) activation up regulates the hepatic expression of apoproteins A-1 and A-11, which in turn promotes increased hepatic HDL secretion (Toth, 2005). The current findings are a promising approach to study changes of HDL levels with respect to various adiponectin polymorphism as well as it could be considered in early protection and management of cardiovascular diseases.

Changes of BMI values are indicated to be significant among comparison of the three groups of genotypes, but it very difficult to speculate and obtain correct decision, since changes are not stratified. Several authors have investigated the impact of rs266729 polymorphism on obesity. Vasseur et al. 2002, Harvest et al. 2004, Karmelic et al. 2012, Wanga et al. 2009, Zadjali et al. 2013, Siitonen et al. 2011, and Suriyaprom et al. 2014, have highlighted significant variation. Sun et al. 2008, Tso et al. 2006, Dolley et al. 2008, Bostrom et al. 2009 and Hsiao et al. 2010 could not observed significant association between obesity and this variant.

Daimon et al. who indicated hypoadiponectinemia to be a risk factor for the development of type 2 diabetes mellitus in Japanese population (Daimon et al. 2003). Baratta et al. and Vendrell et al. also found decreased levels of serum adiponectin concentrations in obese and diabetic subjects and significant inverse associations with some are some possible explanations for the association between T2DM and measures of insulin resistance (Baratta et al. 2004, Vendrell et al. 2004). There serum adiponectin concentrations. The lower levels of adiponectin seen in diabetic patients are believed to be associated with the disorder of metabolism of glucose and lipid in diabetes (Lu et al.2006, Karbowska and Kochan, 2006). Diabetic obese patients demonstrate more deteriorated glucose metabolism exhibited by impaired glucose tolerance, or impaired fasting glucose and had also higher serum free fatty acids, higher total and LDL-cholesterol, higher triglycerides, and lower HDL levels, which could also be contributing factors to the lower adiponectin levels.

Previous reports showed that plasma adiponectin levels are affected by multiple factors including gender, age and lifestyle (Kadowaki etal.2006). In this study we elucidate the impact of gender on adiponectin levels in T2DM and control subjects. Female subjects have significantly higher adiponectin levels compared to male subjects in both T2DM patients with CVD and without CVD was consider as control groups. Our observations of the influence of gender suggest that adiponectin production is also related to factors independent of body weight. At any particular body size or body weight, adiponectin concentrations are greater in women than in men. Further, in men and women pair-matched for age, BMI, adiponectin concentrations were greater in women (Zoccali et al. 2002).

Other factors rather than adiponectin SNPs have been shown to regulate adiponectin levels. A diet rich in whole grain and fat was shown to produce increased adiponectin levels (Mantzoros et al. 2006, Mantzoros et al. 2005). Physical activity was also shown to influence adiponectin, with high levels of physical activity shown to elevate adiponectin levels (Yu et al. 2009). Nelson et al. was reported in which adiponectin levels are altered independently of ADIPOQ polymorphisms after dietary supplementation with a-linolenic acid (Nelson et al. 2007).

Conclusions:-

Adiponectin gene polymorphism rs266729 was associated with CVD in T2DM patients in Al-Najaf Governorate, Iraq. Carriers of the homozygous genotype (GG) have seven fold risk of development of CVD, furthermore the risk in (CG) genotype carriers was two folds. Carriers of (rs266729) polymorphism of adiponectin gene are more prone to the development of cardiovascular diseases and metabolic syndrome by affecting HDL.

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